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Prostate cancer (PCa) is one of most common cancers affecting American men. Radiotherapy is a prevalent modality for the treatment of prostate tumor. Although radiation is capable of eradicating localized prostate tumors, nearly 30% of patients treated with potentially curative doses relapse at the sites of irradiation. Therefore, there is an imperative need to improve the success rate of radiotherapy for PCa. This proposal is focused on a role of 12-lipoxygenase (LOX) in modulating the radiation response of PCa cells. 12-LOX, the enzyme of interest, has already been identified as a promoter for PCa growth and progression. In our studies, 12-LOX was found to promote the resistance of PCa cells to radiotherapy. Inhibition of 12-LOX was found to sensitize PCa cells to radiotherapy and this sensitization maybe due to the activation of caspase-3, suggesting 12-LOX as a novel target for radiosensitization. Further studies will allow us to evaluate 12-LOX as a target to develop radiosensitizer for PCa radiotherapy. The knowledge gained from proposed study will have significant impact on future radiotherapy for PCa.

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INTRODUCTION

Prostate cancer (PCa) is one of most common cancers affecting American men. Radiotherapy is a prevalent modality for the treatment of prostate tumor. Although radiation is capable of eradicating localized prostate tumors, nearly 30% of patients treated with potentially curative doses relapse at the sites of irradiation. Therefore, there is an imperative need to improve the success rate of radiotherapy for PCa.

This proposal is focused on a role of 12-lipoxygenase (LOX) in modulating the radiation response of PCa cells. 12-LOX catalyzes the formation of 12(S)-hydroxyeicosatetraenoic acid (HETE) and it has been implicated in PCa growth and progression. Our studies suggest an involvement of 12-LOX in radioresistance of PCa cells. It is our hypothesis that an increase in 12-LOX expression/activity may lead to an increased resistance in tumors to radiation treatment. Conversely, a downregulation of 12-LOX expression or activity can sensitize PCa cells to radiotherapy. We also hypothesize that VEGF is an important intermediary for 12-LOX mediated radioresistance in PCa. Here we propose to expand our study on the role for 12-LOX in radioresponse in PCa. 12-LOX will be overexpressed in LNCaP and DU145 cells. Then we will study whether an increase in 12-LOX expression in LNCaP and DU145 cells can enhance their resistance to radiotherapy. We also propose to study whether VEGF is required by 12-LOX to enhance PCa radioresistance through blockade of VEGF activity with a neutralizing antibody. Finally, we will evaluate whether BHPP, a 12-LOX inhibitor, can be used to sensitize prostate tumors to radiotherapy. The following specific aims are proposed:

Aim 1. Expand the study on the role of 12-LOX in radioresponse in PCa cells.

Aim 2. Determine whether or not stimulation of VEGF is required by 12-LOX to enhance radioresistance in vitro and in vivo.

Aim 3. Evaluate whether or not 12-LOX inhibitor BHPP can sensitize prostate tumors to radiation in vivo.

BODY OF REPORT

KEY RESEARCH ACCOMPLISHMENT

1 provisional patent application in submission
1 review article published
1 research article in submission
2 abstracts published

PROGRESS

Task 1. Expand the study of the role for 12-LOX in radioresponse in prostate cancer cells. Months 1 - 18:

In this aim, the regulation of 12-LOX levels by IR will be studied in a number of prostate cancer cell lines. The radiosensitizing effects of 12-LOX inhibitors in more PCa cell lines and whether 12(S)-HETE can protect them from radiation will be studied. This task has been largely completed, with findings summarized below.

To study whether or not radiation regulates 12-LOX, we subjected LNCaP cells to radiation of different doses and cultured in serum containing media (RPMI1640-10%FBS) for 16 h. LNCaP cells were selected because they express 12-LOX consistently in culture (Nie et al., 2001). As shown in **figure 1**, low-dose radiation (200 cGy) increased the protein level of 12-LOX, suggesting that the gene expression of 12-LOX was stimulated by low dose radiation. Interestingly, at higher doses (400 and 1600 cGy), the steady state levels of 12-LOX were reduced. The reduction of 12-LOX level is not due to cell death because we did

not notice any significant cell death 16 h after irradiation at doses indicated. The drastic changes in 12-LOX levels as a function of radiation imply that 12-LOX is probably involved in radiation response.

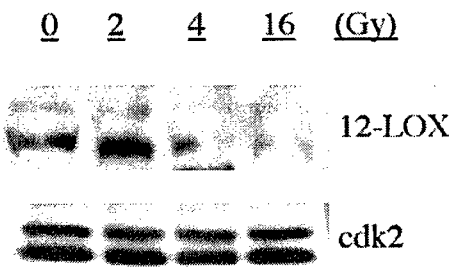


Figure 1. *Effect of radiation on 12-LOX expression in prostate carcinoma LNCaP cells. Note the stimulation of 12-LOX expression by low dose radiation (200 cGy) but at higher doses, 12-LOX expression was reduced (400 cGy and 1600 cGy). The level of cdk2 is included for reference for sample loading.*

To determine whether 12-LOX plays a role in radioresponse of carcinoma cells, we used a panel of PC-3 cell sublines that were stably transfected with an expression construct of platelet-type 12-LOX. The isolated clones had an increased 12-LOX expression and 12(S)-HETE biosynthesis (Nie et al., 1998). Next, we examined the effects of increased expression of 12-LOX on colony formation of carcinoma cells after radiation. As shown in **Figure 2**, nL8, a 12-LOX overexpressing clone (Nie et al., 1998), presented strong radioresistance when compared to its vector control, neo-α (**Figure 2 A**), as indicated by enhanced clonogenic survival. Regression analysis indicated a significant difference in radioresistance between nL8 and neo-σ ($P < 0.01$) (**Figure 2 B**). The data suggest that increased expression or activity of 12-LOX enhances radioresistance in prostate carcinoma cells.

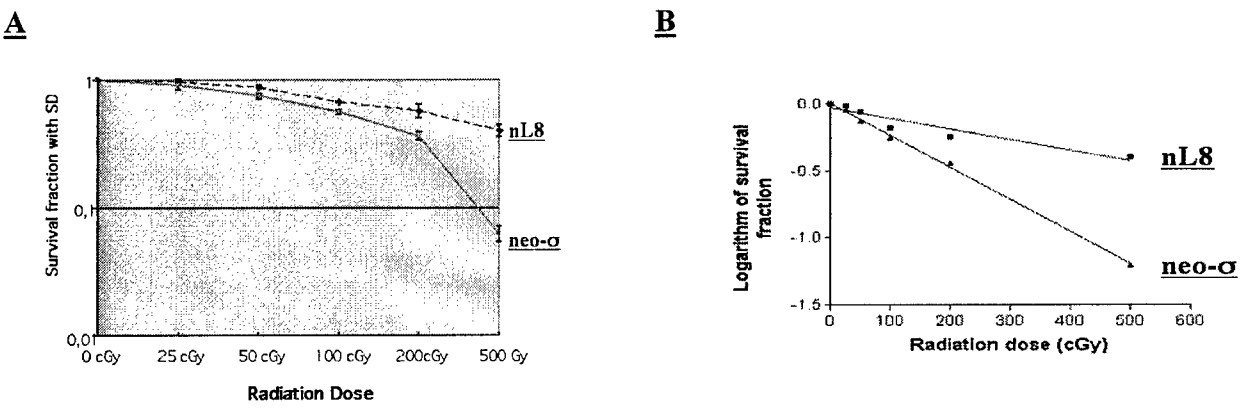
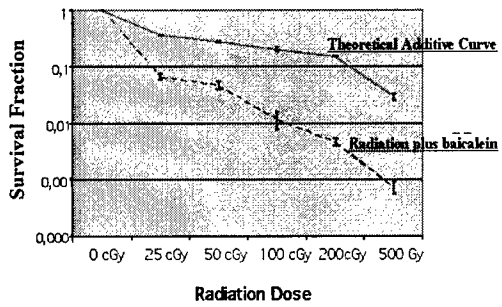


Figure 2. *12-Lipoxygenase enhances radioresistance of PC-3 cells as indicated by colony formation assay. A, Increased clonogenic survival by enhanced expression of 12-LOX in PC-3 cells. nL8, a 12-LOX overexpressing clone of PC-3 cells; neo-σ, vector control. B, Regression analysis. $P < 0.01$.*

Next we studied whether baicalein also sensitizes androgen-independent PCa cells to radiation therapy as it did in LNCaP cells. PC-3 cells were treated with 7.5 μM baicalein for two hours before initiation of radiation. As shown in **figure 3 A and B**, baicalein and radiation, when combined, have super additive or synergistic inhibition on the colony formation of PC3 cells ($P < 0.01$). The data suggest that inhibitor of 12-lipoxygenase also sensitizes androgen independent PC-3 cells to radiation.

A



B

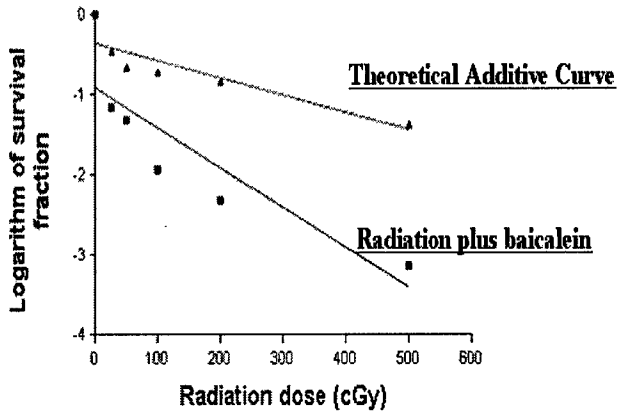
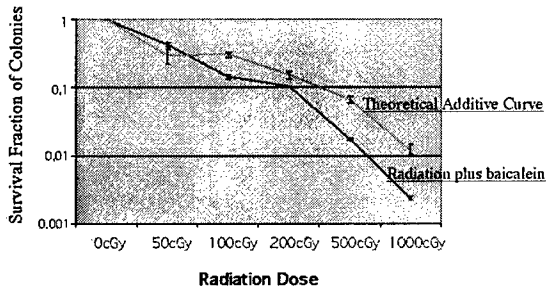


Figure 3. Radiosensitization of androgen independent PC-3 cells by baicalein. ***A.*** 12-LOX inhibitor baicalein sensitizes PC3 cells to radiation as indicated by colony formation assay. ***B.*** Regression analysis. P = 0.0086.

Next we examined whether inhibition of 12-LOX can modulate the radioresponse of PCa cells. First we examined the effect of baicalein, a select inhibitor of 12-LOX, on radioresponse of androgen dependent LNCaP cells. We treated LNCaP cells with 7.5 μM baicalein for 2 hrs before initiation of radiation. As shown in **figure 4 A**, baicalein and IR, when combined, have super additive or synergistic inhibitory effect on the colony formation of LNCaP cells. Regression analysis indicates that combined treatment of LNCaP cells with radiation and baicalein has significant super-additive or synergistic effect ($P < 0.05$) (**Figure 4 B**).

A



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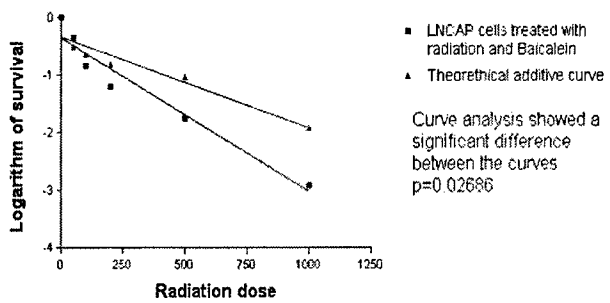

Figure 4. Radiosensitization of LNCaP cells by a 12-LOX inhibitor, baicalein. ***A.*** 12-LOX inhibitor baicalein sensitizes LNCaP cells to IR as indicated by colony formation assay. Refer to the General Method in section D for detailed description of the calculation of theoretical additive curve and other statistical calculation. ***B.*** Regression analysis. P = 0.02688.

The main stable arachidonate product of 12-LOX is 12(S)-HETE. To study whether or not 12(S)-HETE modulates radioresistance of carcinoma cells, we treated PC-3 cells with graded levels of baicalein (0, 3, 6, 9, 12, and 15 μM), in the presence or absence of 300 nM of 12(S)-HETE, for 2 h before irradiation (200 cGy). As shown in **Figure 5 A**, baicalein sensitized PC-3 cells to radiation in a dose dependent manner. The radiosensitization of PC-3 cells by baicalein was completely abolished by exogenously added 12(S)-HETE (**Figure 5 A and B**, $P < 0.01$). Therefore, radiosensitization of PC-3 cells by baicalein is dependent on the *absence* of 12(S)-HETE. The results further suggest the involvement of the 12-LOX activity in radioresistance of prostate carcinoma cells.

A *B*

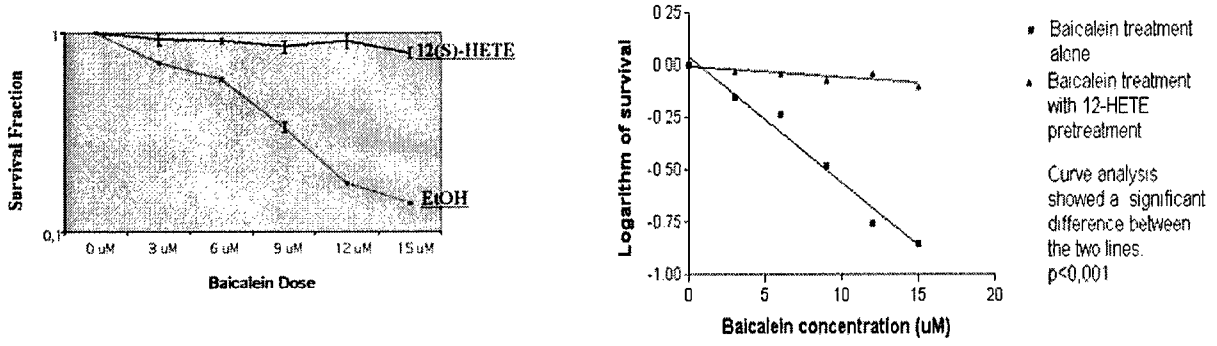

Figure 5. *Radiosensitization of PC-3 cells by baicalein was abolished by exogenously added 12(S)-HETE.* ***A.*** *Attenuation of baicalein radiosensitization of PC-3 cells by 12(S)-HETE as indicated by colony formation assay. The radiation dose used was 200 cGy.* ***B.*** *Regression analysis. $P < 0.001$.*

To study whether 12-LOX inhibitors can also sensitize normal prostate epithelial cells to radiation, we treated human normal prostate epithelial cells (purchased from Clonetics, San Diego, CA) with 7.5 μM baicalein 2 h before radiation (800 cGy). The cells are harvested 36 h after radiation for evaluation of apoptosis using a commercial flow cytometric assay kit based on TUNEL staining (APO-DIRECT, Pharmingen, San Diego, CA). We use apoptosis, rather than clonogenic survival, as the end point for potential radiosensitization of normal prostate epithelial cells by 12-LOX inhibitors. The rationale is that unlike prostate cancer cells, normal prostate cells have limited ability to proliferate and form colonies. As shown in **Figure 6** and **Figure 7**, the presence of baicalein did not potentiate radiation-elicited apoptosis either in normal prostate epithelial cells or in human normal skin fibroblast. The lack of radiosensitization by 12-LOX inhibitor in normal prostate epithelial cells may be due to the low or absence of 12-LOX expression (Gao et al., 1995).

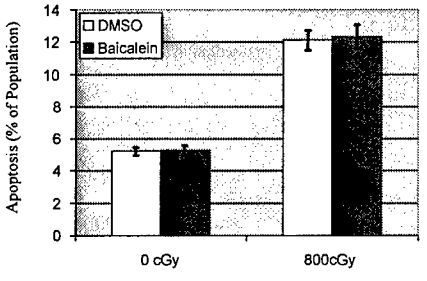

Figure 6. *Lack of radiosensitization of baicalein, a 12-LOX inhibitor, in normal prostate epithelial cells. Note the increase in apoptosis after radiation (800 cGy) and the absence of effect of baicalein treatment on apoptosis, regardless of radiation.*

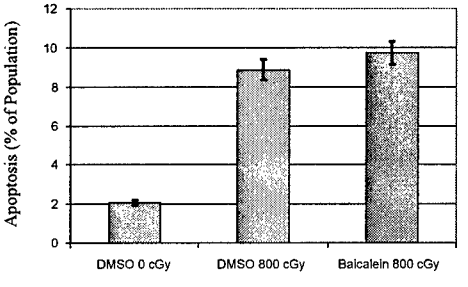

Figure 7. *Lack of radiosensitization of baicalein in normal human skin fibroblast.*

Since 12-LOX inhibitors can induce apoptosis, radiosensitization of tumor cells by baicalein is likely mediated by potentiation of apoptosis. To study this possibility, we evaluated the level of cleaved caspase-3, the activated form of caspase-3. As show in the **figure 8**, combined treatment of A431 cells had highest level of caspase-3 activation.

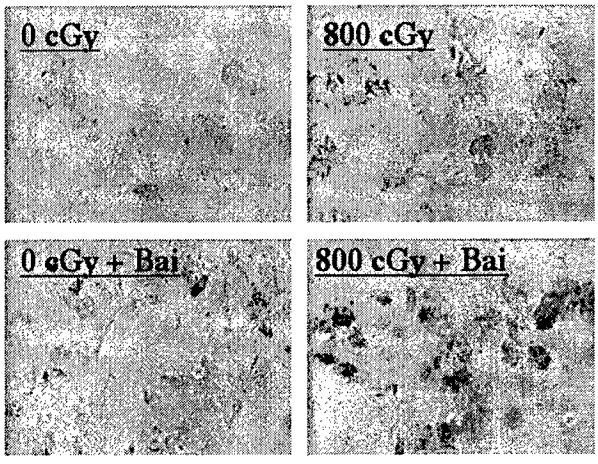

Figure 8. Levels of cleaved caspase-3 16 h after baicalein and radiation treatment. Cells were fixed and immunostained for cleaved (activated) caspase-3 using standard ABC procedure. Blown staining (dark spots if black and white print) indicates positive staining.

Critical for apoptotic processes, caspases are cysteine-dependent and sensitive to oxidation, hence, high levels of lipid peroxide from 12-LOX may lead to their inactivation. To study this possibility, we examined whether 12(S)-HpETE can inhibit the activity of Caspase-3, an effector caspase, which can cleave a broad spectrum of cellular targets. As shown in **Figure 9**, 12(S)-HpETE inhibited caspase-3 activity in a dose-dependent manner.

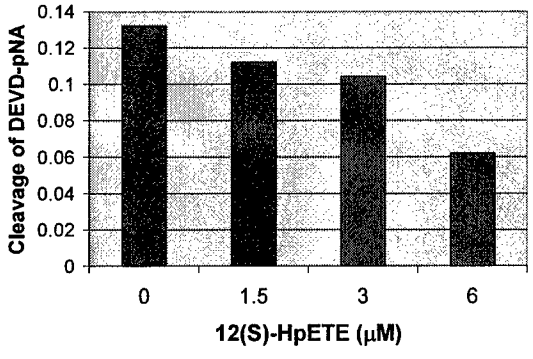

Figure 9. Inhibition of Caspase-3 activity by 12(S)-HpETE. Active caspase-3 (purchased from Biomol) were incubated with graded levels of 12(S)-HpETE for 10 min, before addition of substrate DEVD-pNA. After further 30 min of incubation, the cleavage of DEVD-pNA was measured at 405 nm and expressed as absolute unit. The results represent two independent experiments.

Task 2. ***Determine whether or not stimulation of VEGF is required by 12-LOX to enhance radioresistance in vitro and in vivo.***

We will use a VEGF neutralizing antibody to study whether VEGF is required for 12-LOX mediated radioresistance in PC-3 cells. Matrigel implantation model will be used to assess 12-LOX mediated radioresistance in vivo and to study the role of VEGF in this process. This task has been initiated, with the following preliminary findings:

To study whether 12-LOX can regulate VEGF expression, we measured VEGF levels in culture supernatants from 12-LOX transfected PC-3 cells (nL-8 and nL-12) and their vector controls. As shown in

Figure 10, increased expression of 12-LOX enhanced VEGF expression. Northern blot analysis revealed an increase in the levels of VEGF mRNA in 12-LOX transfected PC-3 cells (nL-2 and nL-8) (**Figure 11**).

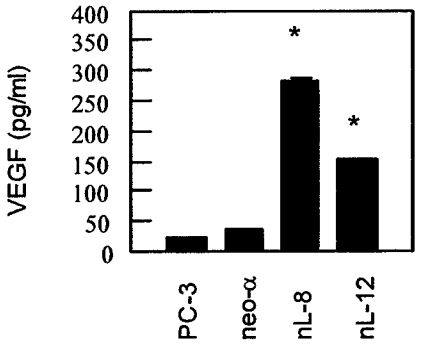

*Figure 10. Increased VEGF Expression in 12-LOX Transfected PC-3 Cells. *, P < 0.01.*

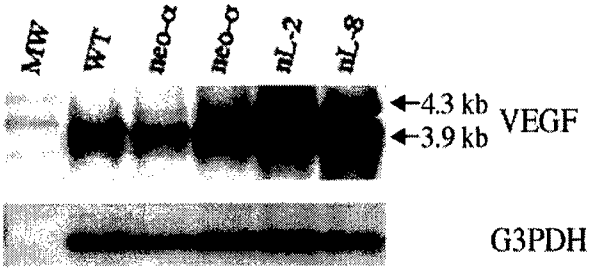

Figure 11. Northern Blot Analysis of VEGF mRNA Levels. Poly(A)+RNA were isolated and the 12-LOX mRNA levels were analyzed with labeled VEGF cDNA. The membrane was then stripped and probed for G3PDH as a loading control.

Studies are ongoing to study whether the stimulation of VEGF expression by 12-LOX can contribute to 12-LOX-mediated radioresistance. The study will determine whether VEGF may play an important role in 12-LOX-mediated radioresistance in vitro and in vivo, through an autocrine loop.

Task 3. ***Evaluate whether or not 12-LOX inhibitor BHPP can sensitize prostate tumors to radiation in vivo.*** We will evaluate whether BHPP, a 12-LOX inhibitor, can be used to sensitize xenografted prostate tumors to radiotherapy.

The task is in planning stage.

SUMMARY/CONCLUSIONS:

Our studies found that 12-LOX promotes the resistance of prostate cancer cells toward radiation treatment. We also found when combined, 12-LOX inhibitors and radiation had synergistic effects in killing PCa cells and this was accompanied by an increase in the level of the active form of caspase-3. Our studies suggest that 12-LOX inhibitors are promising radiosensitizer and further work need to be done to determine the mechanism of radiosensitization and the efficacy of 12-LOX inhibitors in sensitizing prostate tumors to radiation treatment.

REPORTABLE OUTCOMES

- Review article published.
  Nie D, Honn KV.Eicosanoid regulation of angiogenesis in tumors. Semin Thromb Hemost. 2004 Feb;30(1):119-25.
- Research article submitted.
  Nie, D., Y Chen, Y Qiao, A Zacharek, K Tang, J Milanini, G Pages, D Grignon, and KV Honn. Arachidonate 12-Lipoxygenase regulates the expression of vascular endothelial growth factor in a PI3 kinase dependent pathway.
- Abstract published.
  Krishnamoorthy, S., K. R. Maddipati, D. Nie, and K. V. Honn. 12-Lipoxygenase in hypoxia and hypoxia-induced angiogenesis. Proc. Amer. Assoc. Cancer Res. 45: #3591, 2004.
- Abstract published.
  Nie, D., Y. Qiao, A. Zacharek, and K. V. Honn. Blockade of NF-kB sensitizes prostate cancer cells to ionizing radiation. Proc. Amer. Assoc. Cancer Res. 45: #1290, 2004.

- Patent applied. A provisional patent application, entitled "12-Lipoxygenase inhibitors as radiosenstizer for prostate cancer" has been filed.
- Development of animal models: No.